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Detection of microbial trypsin-like enzymes by use of an agar gel

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Abstract

Certain oral micro-organisms associated with periodontal diseases elaborate trypsin-like enzymes. This study describes a simple assay for these enzymes, based on the incorporation of the synthetic substrate N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) into ion-agar gel. The system can provide quantitative or qualitative data within 24 h and is suitable for use in the dental clinic.

Introduction

The progression of destructive periodontal diseases may be significantly affected by oral micro-organisms such as black-pigmented *Bacteroides* species and treponemes. *Bacteroides* species have been recovered in high proportions from patients with advanced periodontitis (Slots, 1979; Tanner *et al.*, 1979). Positive correlations have been established for pocket depth, connective tissue attachment loss, and periodontal indexes in patients with a prevalence of subgingival treponemes (Listgarten and Levin, 1981; Armitage *et al.*, 1982). *Treponema denticola* is a specific spirochete which has been shown to be present in elevated numbers in severe periodontitis (Simonson *et al.*, 1988).

A common characteristic of these micro-organisms, which may enhance their virulence, is that they elaborate trypsin-like enzymes (Slots, 1981; Laughon *et al.*, 1982a). This has also been observed for certain species of *Capnocytophaga*, which are often associated with early periodontitis and have been reported to cause neutrophil and fibroblast dysfunctions *in vitro* (Shurin *et al.*, 1979; Stevens *et al.*, 1980). The trypsin-like enzymes were determined by use of the substrate N-benzoyl-DL-arginine-2-naphthylamide (BANA), a component of the API ZYM system (Analytab Products Inc., Plainview, New York, U.S.A.), which provides a semiquantitative micromethod for measuring nineteen enzymatic reactions. This substrate has been employed qualitatively to identify oral *Bacteroides* spp., *Capnocytophaga* spp. and spirochetes (Laughon *et al.*, 1982b). Recently, Tippet *et al.* (1987) described a method for quantitation of *B. gingivalis* (*Porphyromonas gingivalis*) by counting colonies imprinted on filter paper where their trypsin-like enzymatic activities were revealed by use of a BANA-containing chromogenic system.

Another quantitative laboratory procedure for measuring trypsin-like activity has been developed by Haverback *et al.* (1960) by use of the synthetic substrate, N-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). One advantage of this substrate is that the development of colour from enzymatic action can be monitored during the incubation of a sample. This contrasts with the use of BANA, with which colour development requires the addition of reagents that stop enzymatic action. Preliminary tests of the Haverback BAPNA procedure

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at this laboratory, conducted on twelve strains of *B. gingivalis*, confirmed the sensitivity and utility of the method. We demonstrated that elaboration of trypsin-like activity *vs* time of growth peaked at 48—72 h (Lamberts *et al.*, 1985). However, this laboratory procedure was not convenient for clinical use because of the relatively short shelf life of the substrate in solution, the need for additional reagents and the attention required by procedural details.

The present report describes a simple quantitative BAPNA-in-agar assay which can be employed for measuring trypsin-like activities of oral micro-organisms or applied clinically for tests of human plaque samples. Information from the clinical tests could help to identify patients undergoing changes in disease status or could be useful in monitoring the success of therapeutic measures.

Materials and methods

Preparation of BAPNA-in-agar system

A mixture of 0.10—0.15% BAPNA and 1.0% ionagar was dissolved with continuous stirring at 100°C in 0.1 M Tris HCL buffer, pH 7.7. The temperature was adjusted to 60—65°C and aliquots of the mixture were transferred to vials or microplates and permitted to cool to form gels, which were covered with the buffer to prevent dehydration. The gel containers were sealed with lids or plastic wrap and maintained under refrigeration prior to use.

Micro-organisms and culture conditions

Table 1 lists alphabetically the organisms which were tested for their abilities to elaborate trypsin-like enzyme activity. The bacteria were grown to late log or early stationary phases prior to the tests. The following organisms were grown anaerobically for 72 h in Wilkins-Chalgren broth at 37°C: *Bacteroides asaccharolyticus, Bacteroides denticola, Bacteroides gingivalis, Bacteroides intermedius, Bacteroides loescheii, Bacteroides macacae, Capnocytophaga ochracea* and *Capnocytophaga sputigena.*

Application of samples

Pancreatic trypsin III (Sigma) was dissolved in 0.1 M Tris HCL buffer, pH 7.7, (1 mg/ml dry weight as received). This stock solution was employed to prepare 1.25—1,000 ng/100 μl trypsin standard solutions, which were added to microplate wells containing the BAPNA-in-agar. Plates were read at 405 nm immediately after application (blanks) and following suitable incubation periods.

Micro-organisms were applied to the gels either in culture media or as washed cells, suspended in the Tris HCL buffer or in 0.9% saline. Subgingival plaque samples were obtained from patients by curettes and applied to the gels, either directly or after preliminary suspension in saline.

Results

Linear relationships were found between colour development and the amount of pancreatic trypsin, following 37°C incubations of trypsin in the BAPNA-in-agar system (Figure 1). Extending incubations from 2 to 4 or 6 h increased the sensitivity of measurements while retaining the linearity of the plots of activity

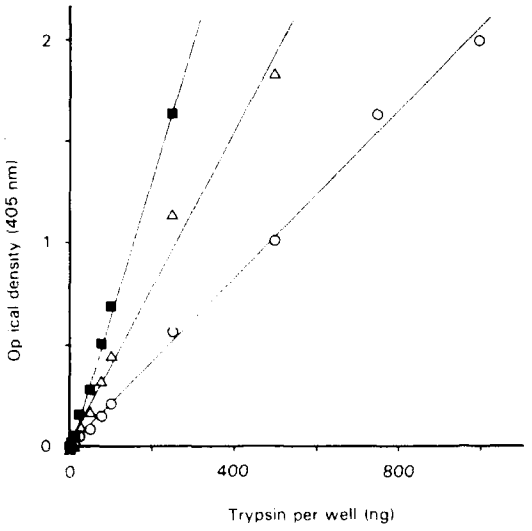

Figure 1 Trypsin standard plots following incubation at 37°C. The incubation times were: 2 h, correlation coefficient 0.997; 4 h, correlation coefficient 0.986; and 6 h, correlation coefficient 0.998.

vs amount of trypsin. Table 1 shows data on trypsin-like activities for sixty-two isolates of oral bacterial species along with information on their Gram-staining properties. It is evident that most of the activity was found with the *B. gingivalis* and *T. denticola* strains. No activity was detectable with the Gram-positive micro-organisms.

Tests of twenty-nine subgingival plaque samples revealed a rough correlation of spirochete level and trypsin-like activity. Twenty samples with high to moderately low spirochete levels showed trypsin-like activity, while nine samples with few if any spirochetes showed no activity.

Discussion

Exploratory tests with the subgingival plaque samples indicated that the BAPNA-in-agar system can serve as a rapid, simple method for detecting microbial trypsin-like activity. The system has several potential advantages for clinical use. (a) Plaque samples can be applied directly to a gel by curette, although precautions must be taken to avoid transfer of gel and buffer to the patient's mouth. This may involve the use of a clean curette for each site sampled. (b) Qualitative or semi-quantitative determinations of activity can be made visually. Representative standards may be prepared in vials by incubating various amounts of trypsin with 0.5—2.0 ml volumes of BAPNA-in-agar, then adding 100 μl of 0.1 N HCl

Table 1 Detectability of trypsin-like activities in oral micro-organisms by BAPNA-in-agar assay

Micro-organism	Gram stain*	Isolate	BAPNA activity
Actinobacillus actinomycetemcomitans	N	ATCC 29523[5]	–
		ATCC 29524[5]	–
Actinomyces naeslundii	P	ATCC 12104[5]	–
Actinomyces odontolyticus	P	ATCC 17982[5]	–
Actinomyces viscosus	P	M 100[5]	–
Bacteroides asaccharolyticus	N	ATCC 25260[5]	–
Bacteroides denticola	N	ATCC 33185[5]	+
Bacteroides gingivalis (*Porphyromonas gingivalis*)	N	D5529 A[4]	+
		D13B 11[4]	+
		D40C 4[4]	+
		D67D 9[4]	+
		D43A 5[4]	+
		D82F 5[4]	+
		376[1]	+
		2A[1]	+
		ATCC 33277[5]	+
		38[1]	+
		JKG 5[2]	+
		JKG 1[2]	+
		D83T 3[4]	+
		3B[1]	+
		D868B 6[4]	+
		D84D 2[4]	+
		JKG 9[2]	+
Bacteroides intermedius	N	ATCC 25611[5]	–
		13043[4]	–
Bacteroides loescheii	N	ATCC 15930[5]	–
Bacteroides macacae	N	ATCC 33141[5]	+
Capnocytophaga ochracea	N	ATCC 27872[5]	–
Capnocytophaga sputigena	N	ATCC 33612[5]	+
Fusobacterium nucleatum	N	ATCC 25586[5]	–
Fusobacterium spp.	N	B[1]	–
		5[1]	–

Table 1 (continued)

Micro-organism	Gram stain*	Isolate	BANA activity
Haemophilus aphrophilus	N	ATCC 13252[5]	–
Lactobacillus casei	P	1[3]	–
		3[3]	–
Leptotrichia buccalis	N	ATCC 14201[5]	–
		ATCC 19616[5]	–
Pseudomonas aeruginosa	N	1[3]	–
Proteus vulgaris	N	1[3]	–
Staphylococcus aureus	P	ATCC 12598[5]	–
Streptococcus faecalis	P	539[6]	–
Streptococcus mutans	P	NCTC 10449[8]	–
		6715[9]	–
		OMZ 176	–
		C-211	–
Streptococcus sanguis	P	ATCC 10557[5]	–
		ATCC 10558[5]	–
		410	–
		Challis	–
Treponema denticola	N	ATCC 33520[5]	+
		D39DP 1[4]	+
		N 39[4]	+
		IPP[4]	+
		Ichelson 2[4]	+
		TRRD[4]	+
		TD 2[4]	+
		Ambigua[4]	+
		D65BR 1[4]	+
Treponema vincentii	N	ATCC 33580[5]	–

* N, Gram-negative; P, Gram-positive.
[1]Forsyth Dental Center; [2]University of Michigan; [3]Naval Dental Research Institute; [4]Virginia Polytechnic Institute and State University; [5]American Type Culture Collection; [6]University of Notre Dame; [7]University of West Virginia; [8]National Culture Type Collection; and [9]National Institute of Dental Research.

to stop the reaction and stabilize colour. (c) Results with plaque or microbial samples can be obtained within 24—48 h. This procedure may also be useful to elucidate the relationship of plaque microbial composition to periodontal disease status.

Data shown in Table 1 agree with previous reports, in which the substrate BAPNA was used to screen for trypsin-like enzymes from *B. gingivalis* and *T. denticola* (Slots, 1981; Laughon *et al.*, 1982a). Further study of pure cultures may provide comparative information on the stabilities and cell-binding properties of trypsin-like enzymes from various micro-organisms.

The BAPNA-in-agar system has demonstrated a shelf life of over 1 year if maintained frozen or shielded from light at 1—4°C, and of over 3 months at room temperature when shielded from light.

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